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(54) Title: POROUS SUBSTRATES FOR DNA ARRAYS

(57) Abstract: The invention relates to a device for use as a support for high density nucleic acid arrays comprising a substrate of inorganic material having a substantially planar porous top surface and a cationic polymer layer bonded to the top surface. Further disclosed is a method for performing a hybridization assay using the substrate of the present invention along with index matching fluids.

POROUS SUBSTRATES FOR DNA ARRAYS

This application claims the benefit of United States provisional patent application 60/152186, filed September 2, 1999.

BACKGROUND OF THE INVENTION

High density arrays are new tools used by drug researchers and geneticists that provide information on the expression of genes . A high density array typically comprises between 5,000 and 50,000 probes in the form of single stranded DNA, each of known and different sequence, arranged in a determined pattern on a substrate. The substrate may be any size, but typically takes the form of a standard 1x3 inch glass microscope slide.

The arrays are used to determine whether single stranded target sequences interact or hybridize with any of the single stranded probes on the array. After exposing the array to target sequences under selected test conditions, scanning devices can examine each location on the array and determine the quantity of target complimentary to its probe . DNA arrays can be used to study which genes are turned on or "up-regulated" and which genes are turned off or "down-regulated". So, for example, a researcher can compare a normal colon cell with a malignant colon cell and thereby determine which genes are being expressed or not expressed only in the aberrant cell. The regulation of these genes serves as key targets for drug therapy.

hybridization of the target to the immobilized probe, as measured by fluorescence emission from the tagged target sequence. The DNA probe material must be retained through a series of washing, blocking, hybridizing and rinsing operations that are commonplace in DNA hybridization assays. Excessive loss of probe DNA leads to low fluorescent signal-to-noise ratio and uncertain or erroneous results.

DNA arrays typically employ a flat, non porous substrate surface made from glass (*see*, e.g. US Patent 5,744,305). Additionally, DNA arrays have for years been printed into organic micro porous membranes such as nylon and nitrocellulose. However, the densities at which one can print DNA solutions into organic microporous membranes is limited because of the tendency for the DNA solution to wick laterally through the membrane causing contamination and cross-talk between adjacent locations.

The present invention proposes to enhance DNA retention for a DNA array through the use of a substantially flat inorganic porous substrate surface. The porous surface increases the density of DNA binding sites per unit area of cross-section, while preventing lateral wicking. The increased number of possible binding sites per unit cross-sectional area in a porous surface ensures greater retention of the immobilized probe DNA.

SUMMARY OF THE INVENTION

The invention relates to a device for use as a support for high density nucleic acid arrays comprising a substrate of inorganic material having a substantially planar porous top surface and a cationic polymer layer applied to the top surface . The porous surface provides increased surface area for the immobilization of DNA probe molecules which results in increased signal during hybridization assays. Further disclosed is a method for performing a hybridization assay utilizing the substrate of the present invention along with index matching fluids.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is a fluorescence scan image of spotted single stranded Cy3 labeled DNA on a porous glass substrate.

Fig. 2 is a fluorescence scan image of Cy5 labeled single stranded DNA that has hybridized to a complimentary immobilized single stranded DNA sequence.

Fig. 3 is a schematic cross-sectional representation of the porous substrate of the present invention with double stranded DNA molecules attached via a cationic polymer.

Fig. 4A is a cross-sectional SEM micrograph of a porous borosilicate binding layer tape casted onto an impermeable calcium aluminosilcate support using a doctor blade gap of 0.0005 inches and fired at 670°C for 2 hours.

Fig. 4B is an elevation SEM micrograph of the substrate of Fig. 4A.

Fig. 5A is a cross-section SEM micrograph of a porous borosilicate binding layer tape casted onto an impermeable calcium aluminosilcate support using a doctor blade gap of 0.0005 inches and fired at 680°C for 2 hours.

Fig. 5B is an elevation SEM micrograph of the substrate of Fig. 5A.

Fig. 6A is a cross-section SEM micrograph of a porous borosilicate binding layer tape casted onto an impermeable calcium aluminosilcate support using a doctor blade gap of 0.0005 inches and fired at 690°C for 2 hours.

Fig. 6B is an elevation SEM micrograph of the substrate of Fig. 6A.

Fig. 7 is a graphical representation comparing spot siz of printed DNA on various substrate surfaces.

Fig. 8 is a graphical representation comparing relative signal of labeled DNA immediately after printing on various surfaces.

Fig. 9 is a graphical representation comparing retention of printed DNA on various surfaces after blocking and hydridizing.

Fig. 10 is a graphical representation comparing the relative signal of printed and hybridized DNA on various surfaces normalized to a glass slide CVD coated with gamma-aminopropyltriethoxysilane.

Fig. 11 is a graphical representation comparing relative hybridization efficiency of printed and hybridized DNA on various surfaces normalized to a glass slide CVD coated with gamma-aminopropyltriethoxysilane.

Fig. 12 is a graphical representation comparing the average background fluorescence on a variety of surfaces at wavelengths of the Cy3 marker on channel 1 (ch1) and Cy5 marker on channel2 (ch2) markers after printing (p), blocking (b), and hybridization (h).

Fig. 13 is a photograph of a calcium aluminosilicate slide, tape cast coated with porous borosilicate glass, and after firing at 670°C for 2 hours. The right side of the slide was infiltrated with refractive index matching glycerol.

Fig. 14A is a scanned image showing fluorescent intensity from a spot of Cy3 labeled DNA on a tape cast porous borosilicate glass slide that was fired at 680°C for 2 hours.

Fig. 14B is a scanned image showing fluorescent intensity from a spot of Cy3 labeled DNA on a tape cast porous borosilicate glass slide that was fired at 680°C for 2 hours, and that has been infiltrated with index matching glycerol.

Fig. 15 is a cross sectional SEM micrograph of a substrate of the present invention having an int rlayer bonding the porous layer to the solid substrate.

DESCRIPTION OF THE INVENTION

Porous Glass

Open celled porous glass having the desirable properties of fused silica has been sold for nearly a half century and is produced by a unique process that circumvents the need for high temperatures in melting and forming. A relatively soft alkaliborosilicate glass is melted in a conventional manner and is then pressed, drawn, or blown into the desired but oversized shape by standard processes used in glass production. The resultant workpiece, which occasionally is given additional finishing operations, is subjected to a heat treatment above the annealing point but below the temperature that would produce deformation. During this heat treatment, two continuous closely intermingled glassy phases are produced. One phase is rich in alkali and boric oxide and is readily soluble in acids. The other phase is rich in silica and is insoluble.

After heat treatment, the workpiece is immersed in a hot dilute acid solution. The soluble phase is slowly dissolved, leaving behind a porous high-silica skeleton. The resulting porous article is commonly known as thirsty or porous glass (Vycor™, code 7930, Corning Inc.).

Pore size distribution in the glass is typically very narrow (± 3 Å from average pore radius). Pore size in the glass varies but generally ranges from 40-50 Å. The pore size may be enlarged up to 200 Å or greater by dissolving the glass with a weakly reactive fluorine-containing compound, for example. Methods of producing porous glasses, typical compositions of porous glasses, as well as properties of commercially available porous glasses are discussed in detail in "Porous and Reconstructed Glasses" Engineered Materials Handbook, Vol. 4, Ceramic and Glasses, p. 427-32, 1992, incorporated herein by reference

In a preferred embodiment, porous glass (Vycor™, code 7930, Corning Inc., Corning, NY) in the form of a substantially flat 1 inch x 3 inch x 1mm slide is used as a substrate for the immobilization of DNA (cDNA or oligonucleotide). The porous glass slide weighs approximately 3 grams and has roughly 7.5 million square centimeters of surface area for DNA attachment. Comparatively, a non-porous glass slide of the same dimensions has only approximately 40 square centimeters of surface area. The porous glass slide offers an approximate 200,000 fold increase in surface area for DNA attachment.

An alternative to the porous glass described above is controlled pore glass (CPG Inc., Fairfield, NJ). Currently, glass beads or granules that attach biomolecules for combinatorial chemistry and various forms of affinity chromatography employ controlled pore glass. Although the lower limit pore radius that can be achieved with controlled pore glass is approximately 75 Å, it is conceived that this type of glass may be employed to form the substantially flat substrate necessary for use as a DNA array substrate.

For the immobilization of biomolecules, and particularly DNA, the porous substrate of the present invention can advantageously be treated with cationic polymer, i.e. polymers having a multiplicity of ionic or ionisable functional groups having a positive charge. Coating a porous substrate with a cationic polymer has several advantages over cationic coatings on flat substrates. First, the porous glass substrate has greater surface area and therefore has more surface exposed silanol groups. Consequently, the negatively charged silanol groups can bind to greater amounts of the positively charged cationic species by electrostatic interactions. This greater density of positive charge leads to greater retention of the negatively charged DNA.

Second, the electrostatic interaction between DNA and cationic polymer coated on a flat porous glass substrate is stronger than the same interaction on a flat non-porous glass substrate because of the greater local availability of silanol groups for electrostatic binding per cationic polymer molecule. Further, the narrow pores (40 Å for Vycor™, code 7930, Corning, Inc.) create an environment of tightly bound water molecul s which lead to lower dielectric

constants. These microenvironments are likely to greatly enhance the strength of the electrostatic int ractions b tween the DNA and silanol groups.

Third, displacement of the cationic polymer from the surface of the porous glass substrate's uppermost surface does not necessarily lead to complete displacement of the polymer from the substrate. As shown in Fig. 3, the cationic polymer 12 may remain attached to the substrate 10 by tail attachment within a pore 14 even if it is displaced from the surface 16. It is also thought that this type of dynamic equilibrium may enhance hybridization by serving to extend the DNA 18, which interacts with the polycation 12, away from the substrate surface 16 and further into solution.

The cationic polymer coated porous glass substrate is compatible with existing protocols for retention of probe and hydridization of target DNA. For example, the spotting of probe DNA should preferably be followed by blocking of the substrate surface with succinic anhydride (or anionic polymers such as poly(glutamic acid), poly(acrylic acid), anionic dendrimers, heparin, etc.) that would confer a net negative charge on the unspotted surface. This blocking step has the effect of reducing any non-specific binding of the highly negative target DNA sequence to the unspotted regions.

A non-inclusive list of examples of cationic polymers suitable for use as coatings for the porous glass substrate include: polylysine and the corresponding copolymers with neutral amino acids, polyethyleneimine, polybrene, amino silanes such as gamma aminopropyltriethoxysilane (GAPS), cationic dendrimers, and polyvinylamine.

Tape Cast Porous Layers

Another viable method for producing a porous substrate for a DNA array is for the production of a porous DNA binding surface by tape casting of glass or ceramic particles onto a dense backing. The dense backing may take any form, but is preferably a high melting temperature material such as calcium aluminosilicate (Corning Inc., code 1737). The size and amount of porosity in a tape cast layer can be controlled by the solids loading of the slip, firing temperature and time, and size of th ceramic or glass particles in the slip. Typical values of porosity rang between 0 and 70 p rcent, and size of the

pores can be varied between 0.1 to 20 μm. Thickness of the layer is controlled by the gap height of the tape casting blade. Tape casting is an attractive process for manufacture of porous DNA binding layers for several reasons: a large scale, continuous, manufacturing process is easily implemented; tape cast layers have a uniform thickness, and the process as a whole is reproducible; cost of chemicals used in the manufacture of the slip is low; and tape casting is capable of producing layers with thickness in the range 5-100 μm in a single step. Ease of manufacture, uniformity and reproducibility of layers from batch to batch or piece to piece is higher for tape casting than comparable techniques such as sol-gel, spray coating, or dip coating.

One potentially adverse effect from a porous DNA binding layer is light scattering. Light scattering due to the difference in refractive index between the pore and the solid material is most severe when the pore size is similar to the wavelength of the fluorescent markers or excitation source. The chemical markers fluoresce in the visible range, 300-800 nm, which includes the size of pores inherent to the tape cast porous layers. Light scattering is expected to be a problem, but it can be reduced or nullified. First, the solid component of the porous layer when fully dense should be transparent to the fluorescent light of the chemical tag in the absence of porosity. Second, a transparent material with the same refractive index as the solid component of the porous layer could be infiltrated into the pores after the DNA hybridization step. The infiltrant must not contain any impurities that fluoresce such as aromatic groups, and it must not be fluorescent itself. Ideally, the porous layer would be transparent after infiltration and no light scattering should be detected. In one embodiment of this invention, a transparent solid such as boroaluminosilicate glass is employed as the porous layer and an index matched material such as a synthetic, amorphous wax or glycerol is infiltrated into the pores after hybridization to minimize light scattering. For example, a carbonyl monomer such as methylmethacrylate could be infiltrated into the pores and then polymerized. Polymerization of methylmethacrylate forms the polymer polymethylmethacrylate which has an index of refraction of ~1.50, nearly identical to borosilicate.

In yet another embodiment of this invention, light scattering may be used advantageously to increase the probability of excitation striking fluorophor. Excitation is scattered multiple times before exiting the porous coating, whereas on a flat slide, the excitation has but one opportunity to interact with a fluorophor. Modifications to scanning equipment that consist of optical filters of switched detectors may be used to prevent scattered excitation from reaching the detector.Porous Substrate Formed From Sol-Gel Process

US Patent 5,585,136, incorporated herein by reference, describes a method for producing a porous coating made from sealed frit using a sol-gel coating process. This is achieved by mixing an appropriate organo-metallic sol-gel solution with up to 90% by weight of the selected inorganic powder material chosen for the make-up of the porous layer. The solution is applied to a solid support either by spraying, or more preferentially, by dipping. Next, the piece is fired in order to produce a crack free layer. This process may be repeated several times in order to achieve a particular desired thickness and in order to eliminate the potential for cracking.

This process for creating a porous substrate for DNA array has several advantages. First, different powders (and particularly powders containing a high level of silica) may be employed because the bonding properties are not determinant on the consolidation of the powders, but rather from the firing of the organo-metallic batch element. Second, this process can operate at low temperatures, thereby enabling the use of soda-lime glass slides as the support material. Third, porosity can be tightly controlled by the frit particle size distribution, the sol-gel formulation, and frit/sol-gel/solvent ratio.

Although the following examples illustrate the use of the present invention with DNA, it is contemplated that the porous inorganic substrate may be used as a substrate for the immobilization of arrays of other biomolecules or "binding entities" that have a specific affinity for another molecule through covalent or non-covalent binding. Preferably, a specific binding entity contains a functional chemical group (primary amine, sulfhydryl, aldehyde, etc.), a common sequence (nucleic acids), an epitop (antibodies), a hapten, or a ligand that allows it to covalently r act or non-covalently bond to a common

functional group on the surface of the inorganic porous substrate of the present invention. Specific binding entities include, but are not limited to: deoxyribonucleic acids (DNA), ribonucleic acids (RNA), synthetic oligonucleotides, antibodies, proteins, peptides, lectins, modified polysaccarides, synthetic composite macromolecules, functionalized nanostructures, synthetic polymers, modified/blocked nucleotides/nucleosides, modified/blocked amino acids, fluorophores, chromophores, ligands, chelates and haptens. The term "biomolecule" and "binding entity" are interchangeable for purposes of this disclosure.

Although the invention is described above and in the following examples for purposes of illustration, it is understood that such detail is solely for that purpose and variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention which is defined by the claims which follow the examples.

Example 1 – Porous Glass.

1 inch x 3 inch x 1mm porous glass slides (Vycor™, code 7930, Corning, Inc.) were coated with gamma-amino propyltriethoxysilane (GAPS) using a 1.0% aqueous solution of GAPS at a pH of 4.0 adjusted by using acetic acid. The coating was accomplished by immersing each slide in the silane solution for 30 minutes. The coated slides were then washed thoroughly with distilled water and dried. The slides were then heated to 160° C in order to cross-link any free silanol groups.

Next, a water and glycerin solution containing Cy3 labeled single stranded 80-mer oligonucleotides in a concentration of 1 picomole oligo per microliter was printed onto the slide surface by micropipette. The printed slide was heated in humidity for one hour at 55°C. The slides were then washed twice with 5 x SSC and 0.1% SDS at 55°C to remove any unattached or unbonded DNA. The slides were then treated with a solution of succinic anhydride dissolved in DMF for blocking.

The blocked slides were next hybridized with the Cy5 labeled compliment to the immobilized oligo at 55°C using a probe-clip press seal

incubation chamber in hybridization buffer (Boehringer Mannh im, cat. # 1717473). Th slides were th n scanned using a fluorescence det ction scanner (Scan Array 3000, General Scanning Inc.). The slides were scanned twice. First, the slides were scanned for Cy3 prior to hybridization with the complimentary oligo. Then, slides were scanned for Cy5 after hybridization.

Results

Fig. 1 shows a Cy3 scan of the sample slide 2 prior to hybridization, and after the two washings with 5X SSC - 0.1% SDS at 55°C. As shown, the signal strength from the four spotted regions 5, indicates significant oligo immobilization.

Fig. 2 shows a Cy5 scan of the sample slide 2 after hybridization with the complimentary labeled oligo. As shown, the signal strength from the spotted regions 5 indicates detectable hybridization which must indicate continued retention of the probe to the porous glass, even after blocking and washing steps.

Example 2 – Tape Cast Porous Layers

Borosilicate glass (Pyrex code 7761, Corning Inc.) was selected as for use as the porous layer for three reasons: 1) it is transparent; 2) borosilicate glass slides are readily available for use as the substrate; 3) the glass transition/sintering temperature of the substrate and porous layer should be similar to provide for strong adhesion to the slide. At this point, one other criteria can be stipulated for porous binding material. In the ideal case, the surface should be positively charged in a neutral aqueous solution to aid in attachment of the negatively charged DNA molecules.

Borosilicate Powder

Crushed borosilicate glass that had already been sieved to 400 mesh was wet-milled to reduce particle size. The ball mill consisted of a one gallon bottle (Nalgene) charged with 500 g of the crushed borosilicate glass, 7.5 kg of 1 cm ZrO_2 milling cylinders and was filled to 85 percent of full with isopropanol.

The ball mill container was rolled at ~2-3 rotations per second for 24 hours. After milling, the liquid slurry was poured from the one gallon bottle into a 2 L beaker, and the isopropanol was evaporated by heating on a hot plate to recover the borosilicate. The slurry was stirred while drying with a magnetic stir bar to prevent particle settling. The obtained powder was in the form of a hard, agglomerated cake. Formation of the hard agglomerates during drying is believed to result from leaching of alkaline components from the glass by isopropanol. The leached alkalines behave in a cementatious-like manner upon loss of the isopropanol solvent and bind the borosilicate particles together. The mean particle size of the milled borosilicate powder was measured to be 3.5 μm. Use of non-leaching solvent during milling might lead to smaller average particle size. Despite powder agglomeration, this borosilicate was used in preparation of slip for tape casting.

The Slip

US Patent 5,089,455, incorporated herein by reference, gives a detailed description of the preparation of zirconia based slips for the tape casting of thin zirconia electrolytes such as for fuel cell applications. Preparation of the borosilicate slip for casting of a porous DNA layer was performed roughly according to the procedure given in that patent. The recipe was adjusted to account for the difference in density of ZrO_2 and borosilicate, and no settling was performed to narrow the particle size distribution. In brief, 100 g of milled borosilicate powder, 90.9 g ethanol, 21.98 g 1-butanol, 5.0 g propylene glycol, 6.25 g distilled water, 2.5 g hydrogenated glyceride phosphate (Emphos), and 1125 g of one cm ZrO_2 milling balls were weighed into a 500 ml bottle (Nalgene) and vibratory milled for 72 hours. The milled slip was poured from the 500 ml bottle without the milling media into a new 250 ml bottle (Nalgene). The final step in the preparation of the slip was to add 5.0 g of a 50 w/o mixture of glacial acetic acid and isopropanol, 8.75 g dibutylphthalate, and 15 g polyvinylbutyral, and five or six 1 cm zirconia milling balls. The bottle was then rolled gently at <1 rotation per second to thoroughly mix and remove bubbles for at l ast 72 hours prior to tape casting.

Casting and Firing

A sheet of polyester film (Mylar, Dupont, Wilmington DE) was cut to just cover the surface of a small vacuum table of a small tape caster (Pacific Scientific, Gardner/Neo Tec. Ins. Div., Silver Spring, Maryland) normally used for tape casting of ZrO_2. The size of the vacuum table is approximately 12 x 9 inches. Rows of double stick tape spaced 0.25 inches apart were applied parallel to the long direction of the table onto the mylar. Slides of calcium aluminosilicate (Corning Inc., code1737) glass were then tiled onto the Mylar, the double stick tape functions to hold the slides tightly in place during casting. After the entire surface of the air table was tiled with glass slides, the surface of the slides and the tape casting blade were cleaned using a lint free cloth and ethanol. A doctor blade with a gap height of 0.0005 inches was selected to give a porous borosilicate layer of approximately 6-12 μm in thickness. A pipet was used to draw 10 ml of the borosilicate slip, and the slip was applied in front of the doctor blade. The blade was pushed across the slides at a rate of 0.2 feet per second. The coated slides were allowed to dry as exposed to the atmosphere for 30 minutes. Coated slides were fired at temperatures of 670, 680, 690, and 700°C for 2 hours.

DNA Printing and Analysis

Slides measuring 1 inch by 3 inches with a thickness of ~1 mm with various types of surfaces such as porous borosilicate, porous glass (Vycor, Corning Inc.), and flat glass were aminated using gamma-aminopropylsilane (GAPS). GAPS was applied to the slides by either chemical vapor deposition (CVD) or dip coating. For dip coating, slides were immersed in GAPS solution for a length of time that is chosen based upon the nature of the surface. Porous slides such as Vycor may be left in the GAPS solution for over 12 hours, while flat glass slides were coated for ~30 minutes. After GAPS coating, whether dip or CVD, slides were dried at 120°C for 1 hour. In order to ensure cleanliness, slides were covered during drying by a glass evaporation dish.

Printing of DNA was performed either by manually pipeting DNA onto a slide or using a robotic instrument. Preparation of the printing solution started with 8.3 µg of PBR-322 DNA tagged with the fluorescent Cy3 marker in 200 ml of water. The DNA contained 1500 nucleotide base pairs. The solution was vacuum centrifuged down to a volume of 45 µl at a concentration of 0.184 µg/µl. The final DNA printing solution was obtained by diluting this solution with distilled water to a DNA concentration of 0.125 µg/µl.

For robotic printing, 25 µl of the DNA solution was placed in a 384 well plate to feed the print head. The robot was programmed to print a 5 by 5 grid of microdots onto the slides spaced 500 µm apart (center-to-center). The diameter of the print pins used was 200 µm, and the volume of DNA solution printed for each microdot was 200 nl. Print pins were rinsed between printing of each spot according to the following procedure: 1) water bath for 3 seconds, 2) sonic water bath for 5 seconds, 3) heated drying for 3 seconds, and 4) air dry for 2 seconds. After printing, the slides were subjected to the following treatments:

1) Scanned to verify that DNA was printed and to establish an initial printed DNA concentration.
2) Exposed to 95°C water.
3) Binding sites not holding DNA were blocked by 5% succinic anhydride in dimethylformamide (DMF).
4) Bound DNA was denatured in 95°C water for 2 minutes, quenched in ethanol, and blown dry in nitrogen.
5) Scanned again to measure any loss of printed DNA associated with steps 1-3.
6) Hybridized with complementary DNA under cleaned cover slips with 22 µl of solution in plastic 2 slide containers sealed with parafilm in 42°C water bath overnight (~18 hrs). DNA for hybridization was supplied by Santona Pal, and was tagged with Cy5 fluorescent marker. The solution as supplied was buffered

with 5X SSC, 0.1 % sodium dodecylsulfide (SDS), and 25% formamide, and concentration of the DNA was $5.7X10^{-8}$ M.

7) Cover slips were removed in solution of 1X SSC and 0.1% SDS.

8) Washed in a stirred solution of 42 C 1X SSC and 0.1% SDS for 1 hour.

9) Washed in a stirred solution of 42 C 0.1X SSC for 1 hour.

10) Blown dry with nitrogen gas.

11) Scanned for detection of printed and hybridized DNA.

A fluorescence detection scanner (ScanArray 3000, General Scanning) was used to detect fluorescence from the Cy3 and Cy5 markers on the printed and hybridized DNA, respectively. The scanner was setup to detect Cy3 on channel 1 and Cy5 on channel 2. The following procedure was used to scan slides after printing in step 1:

1) Quick scan a large area containing the spots at using a laser power (LP) of 95% to excite the fluorescent tags. The photomultiplier tube (PMT) threshold for detection of fluorescence was set at 95% on channels 1 and 2.

2) Auto range channel 1 to the area containing the printed DNA spots at 85 LP, rescan at high resolution using LP 85 and the auto ranged PMT setting.

3) After all the slides have been scanned rescan the slides at the lowest auto ranged PMT setting on channel 1.

The scanning procedure used after blocking and washing, steps 3 and 4, was as follows:

1) Quick scan a large area containing the spots at setting of 95 LP and 95 PMT on channels 1 and 2.

2) Rescan each slide on chann l 1 at 85 LP, and the autoranged PMT valù after printing.

After Hybridization, steps 6 through 10:

1) Quick scan a large area containing the spots at 95 LP and 95 PMT on channels 1 and 2.
2) Scan each slide on channel 1 at 85 LP, and the autoranged PMT value after printing.
3) Scan each slide on channel 1 at 85 LP, and the lowest PMT value found after printing.
4) Auto range channel 2 to the area containing the spots at 85 LP, rescan at high resolution using LP 85 and the auto ranged PMT setting.
5) After all the slides have been scanned, rescan the slides at the lowest auto ranged PMT setting on channel 2.

Results Fired slides are translucent and have a hazy appearance due to light scattering. In general, the porous borosilicate layers are strongly bonded to the calcium aluminosilicate glass substrate, and porosity of the coatings decreases with increasing firing temperature. Firing of borosilicate at temperatures above 720°C resulted in nearly fully dense layers. FIGs. 4-6 show SEM micrograph of the cross-sections (a) and surface (b) of a porous borosilicate DNA binding layer tape cast with a doctor blade with a 0.0005 inch gap after firing at 670 (FIG.4), 680 (FIG.5), and 690°C (FIG.6) for 2 hours, respectively. Morphology of the porosity varied considerably as a function of sintering temperature. Borosilicate particles 20 appeared as isolated independent entities in porous layers fired at 670°C as shown in FIG. 4A and 4B. Porous borosilicate layer 20 is attached to the impermeable calcium aluminosilicate support 22. At 680°C, the particles were joined to one another by well-formed necks 24 as shown in FIG. 5A, and there appeared to be a bimodal distribution of pore size as shown in FIG. 5B. Again, the porous borosilicate layer 26 is attached to the impermeable calcium aluminosilicate support 28. Larger pores were ~5 μm,

and the smaller pores have an average size of ~0.5 μm. At 690°C, as shown in FIG. 6A and 6B, th outer surface of the porous layer 30 is nearly fully dense, but there are isolated channels 32 approximately 1 μm in diameter that connect to a subsurface region of higher porosity. Again, the porous borosilicate layer 32 is attached to the impermeable calcium aluminosilicate support 34. The thickness of the films was measured to be 10-12 μm for firing temperatures of 680 and 690°C and 18-20 μm at 670°C. Clearly, the number of possible DNA binding sites is higher in a tape cast porous layer as compared to a plain flat glass slide.

DNA analysis for assessment of the performance of DNA binding surfaces is quite simple. Slides with different surfaces were printed with DNA tagged with a fluorescent marker. Single stranded DNA complementary to the printed strand and tagged with a different fluorescent marker was used for hybridization. The entire printing, blocking, hybridization procedure is described above. Processed slides were analyzed using a scanning instrument capable of exciting the fluorescent tags and detecting any resulting fluorescence. The analyzer scans the slide on a pixel-by-pixel basis, a laser excites the tags, and a photomultiplier detects fluorescence of the tags. The instrument builds images from the fluorescent signal of the printed and hybridized strands of DNA using color to represent intensity. Image analysis software is used to integrate signal intensity over the region where DNA was printed and to measure average background intensity where DNA was not. Background signal originates from light scattering events, nonspecific binding of DNA, or fluorescence from impurities and may be treated as noise. Average background signal is subtracted from the signal measured at locations where DNA was printed to arrive at the signal due to fluorescent tags on printed or hybridized DNA alone. Signal of the DNA is representative of the absolute quantity of printed and hybridized DNA that remain after washing, blocking, hybridizing, and rinsing operations. The signal-to-noise ratio is defined as the signal due to the DNA alone divided by the average background. Comparison between signal from printed DNA to the hybridized compl mentary DNA is useful, as well. If signal from the printed DNA is dramatically higher than for

the hybridized complementary strand, hybridization time may be too short or access of the complementary strand to the printed DNA may be restricted. On the other hand, there is likely a problem with blocking or rinsing procedure if signal from the hybridized DNA is higher than for the printed DNA. It must be noted here that fluorescence efficiencies of tags on the printed and hybridized DNA are not identical, and one-to-one comparisons should not be made. Nevertheless, standards may be defined for weighting of the results to facilitate comparison.

In the first evaluation, the performance of a porous borosilicate coated calcium aluminosilicate slide fired at 700°C for 2 hours was compared to a flat glass slide and to a porous glass (Vycor, Corning Inc. code 7930) slide. The DNA printing and hybridization procedure was identical for all three types of slides with the exception of GAPS coating. The flat slide was coated using a CVD method, and the Vycor and tape cast porous borosilicate slides were dip coated. Table I summarizes the results. The Vycor glass slide gives the highest signal-to-noise ratio for printed DNA, however, signal-to-noise ratio for the hybridized strand is lower than the other two types of slides by a factor of 6. The signal-to-noise ratio obtained for the printed DNA on the porous borosilicate slide was less than for the Vycor by only 0.25%, and it had the highest signal-to-noise ratio for hybridized DNA. Comparison of absolute signal was also made by normalizing with respect to the flat slide and is given in the second two columns of Table I. The porous borosilicate coated slide gave the greatest absolute signal due to both printed and hybridized DNA. Absolute signal from printed DNA on Vycor is also quite high, but the absolute signal from hybridized DNA is a factor of 5 lower than for the flat slide. It is believed that on Vycor, DNA molecules pile on top of one another with the result that the most of the DNA is not accessible for hybridization. The tape cast porous borosilicate is superior to Vycor and flat glass slides in terms of absolute signal in general, and it can be concluded that porous borosilicate retains the greatest quantity of DNA overall. Signal-to-noise ratio for the porous borosilicate can likely be further improved by index matching to reduce light scattering. This will be discussed later. Lastly, th efficiency of

hybridization was assess d by comparing the ratio of absolute signals of printed and hybridized DNA. Flat glass slides gave the greatest degree of hybridization per printed DNA molecule by a factor of three. In absolute terms, however, the porous borosilicate retained the most printed DNA and held more hybrid DNA strands. Access of hybridized DNA to the printed DNA in tape cast porous borosilicate may be improved by increasing porosity.

In another set of experiments, tape cast porous borosilicate was compared to flat and sol-gel coated slides. In all, six types of slides were prepared and printed with DNA using the robotic instrument in the order given in the following list:

1) Flat slide, GAPS coated by CVD made at FRC
2) Flat slide, GAPS coated by dipping
3) Sol-gel coated, and GAPS coated by dipping
4) Porous borosilicate fired at 690°C for 2 hours, GAPS coated by dipping
5) Porous borosilicate fired at 680°C for 2 hours, GAPS coated by dipping
6) Porous borosilicate fired at 670°C for 2 hours, GAPS coated by dipping.

Twenty five microdots were printed on each slide, and each dot was analyzed individually to ensure that observations were supported statistically.

Spot size was observed to be a function of surface type as is shown by FIG. 7. Spot sizes of the two flat slides were about 260 μm in diameter, somewhat larger than the print pins (200 μm). Spot size on the sol-gel coated slide was about 290 μm. The largest spot sizes were obtained for DNA printed on tape cast porous borosilicate, and spot size was observed to increase with sintering temperature. If the volume of DNA solution printed is constant, spot size intuitively increases with sintering temperature since less porosity is available per unit volume. Spot size can be controlled by adjusting the thickens of the porous borosilicate layer, a thicker layer should result in a smaller spot size.

Fig. 8 is a plot of the relative fluorescent signal measured immediately after printing normalized to the CVD GAPS coated flat slide. Average signal for each spot was calculated within 450 μm diameter circle. Porous borosilicate fired at temperatures of 670 or 680 °C gave higher signal by nearly an order of magnitude compared with sol-gel and flat glass slides. The high signal intensities for porous borosilicate may be due to a number of factors such as 1) self quenching of fluorescence on flat and sol-gel coated slides due to excessive surface DNA concentration, 2) changes in DNA concentration in the printing solution over time due to evaporation, 3) misfocus of the analysis instrument at a point below the slide surface, or 4) capillary forces pulling more DNA from the print pin into the porous borosilicate. Retention of printed DNA after blocking and hybridizing was assessed by taking the ratio of the average fluorescent signal intensity within a 220 μm diameter circle inside each spot to the same spot immediately after printing. FIG. 9 shows a plot of percent retention for each slide with exception porous borosilicate slide fired at 690 °C. Retention for that slide was greater than one hundred percent. Error bars in the plot are the spot-to-spot standard deviation. Porous borosilicate fired at 670 °C was found to retain the greatest quantity of printed DNA after blocking and hybridizing.

Hybridization efficiency after completion of all processing was determined by taking the ratio of fluorescent signal from the hybridized DNA (Cy5 tagged) to the printed DNA (Cy3 tagged). FIG. 10 is a plot of the relative signal, and FIG. 11 is a plot of the normalized hybridization efficiency. Data in both figures were normalized with respect to the flat CVD GAPS coated slide. Fluorescent signal from printed and hybridized DNA was highest for porous borosilicate fired at 670 °C by a significant margin over flat or sol-gel coated slides. Hybridization efficiency was highest for porous borosilicate fired at 680 °C. Unexpectedly, the flat slide dip coated with GAPS had next highest hybridization efficiency, but the other porous borosilicate slides were only marginally inferior. In general, these results agree with initial evaluations comparing porous borosilicate to Vycor flat slides as described above. Sol-gel

coated and Vycor slides have v ry small porosity and retain printed DNA, but the printed DNA is not accessible for hybridization.

Background signals for the slides were compared after printing, blocking, and hybridizing steps at fluorescent wavelengths corresponding to the Cy5 and Cy3 markers. The results are plotted for each type of slide in FIG. 12. Values for background intensity were obtained by averaging over a circle with a diameter of 1100 μm at location far from the printed DNA spots. As expected, background intensity was highest for porous borosilicate and increased with decreasing sintering temperature, i.e. increasing porosity. Despite the higher background, the signal-to-noise-ratio for the porous borosilicate was superior to flat glass or sol-gel coated slides. FIG. 12 is plot of the signal-to-noise (signal-to-background) ratio for the slides with error bars used to indicate the spot-to-spot standard deviation.

Light scattering in porous borosilicate DNA binding layers which gives high background can be reduced by infiltration of an index matched material into the pores. This is most easily accomplished using a fluid. Glycerol was selected since it's index of refraction of 1.48 is a close match to the index of refraction of borosilicate, 1.50. FIG. 13 shows an optical photograph of a tape cast porous layer made on a calcium aluminosilcate support and fired at 670°C for 2 hours 40. The left-hand side 42 appears hazy due to light scattering, but the right hand 44 was infiltrated with a drop of glycerol and is transparent. In the figure, the drop of glycerol was covered with a glass cover slip to spread the drop, hinder evaporation, and prevent leakage. A spot of 0.4 μl of Cy3 labeled DNA was deposited onto a tape cast porous borosilicate slide that was fired at 680 °C for 2 hours. After drying, a small drop of glycerol was placed on the same spot and covered with a cover slip. The background for Cy3 fluorescence decreased by more than a factor of ten. FIGs.14A and 14B show scanned images of the printed spot of DNA before infiltration (FIG. 14A) and after (FIG. 14B). Brighter contrast indicates greater signal intensity. Notice that the background 52 surrounding the printed spot 50 is darker after infiltration. Tape cast porous borosilicate of release code 7761 on calcium aluminosilicate (code 1737) glass slides has been shown to retain th greatest

absolute quantity of DNA after printing and through all washing, blocking, hybridizing, and rinsing processes. Printed DNA bound on porous borosilicate is accessible for hybridization, and signal-to-noise ratio and absolute signals are higher than for porous Vycor, flat glass slides, or sol-gel coated slides. The optimum firing temperature for this borosilicate composition lies between 670 and 680 °C. Index matching was demonstrated to be effective for reduction of light scattering by porosity in the porous borosilicate layer. Novel aspects of this invention are as follows:

1) The use of tape casting, screen printing and spray coating for production of porous DNA binding layers with enhanced number of binding sites.
2) Use of a transparent (in the fully dense state) solid such as borosilicate to form the porous layer.
3) Use of a solid with a positive surface charge to bind the negatively charged DNA.
4) Infiltration of an index matched material into the porosity of the tape cast layer to lower light scattering.
5) Addition of Ag or other metals capable of forming strong bonds with DNA.
6) Focusing of the fluorescence detector to a plane that lies exclusively within the tape cast porous layer.
7) Use of a porous layer to improve printing uniformity and repeatability through control of spot size and solution volume.

Slide	Printed SN	Hybridized SN	Printed S/S_{flat}	Hybridized S/S_{flat}	$S_{hybridized}/S_{printed}$
Tape Cast	1365	290	8.9	2.65	1.12
Vycor	1400	32	5.4	0.2	0.13
Flat	1100	200	1.0	1.0	3.8

Tabl #1: Comparison of signal-to-noise ratio (SN), and normalized absolute signal of printed and hybridized DNA on porous tape cast borosilicate, Vycor, and a flat glass slide.

Glass Interlayer

In another embodiment, a glass interlayer may be used to enhance the bonding of the porous layer to the glass substrate. The glass used to form the interlayer preferably will have a softening point that is lower than the glass used to form the porous layer. Firing at a temperature that will partially sinter the porous layer should yield a nearly fully dense bonding layer with many strong attachments to the porous layer. Further, use of an interlayer adds chemical durability to the porous substrate.

The interlayer or bonding layer may be manufactured by green-on-green or green-on-fired methods. For example, tape casting slips of both glasses should be prepared as described above. The slip that contains the glass frit for the interlayer could be tape cast onto a glass panel with a tape casting blade with a gap height of 0.5 mil. The green-on-green body can then be fired at the appropriate temperature. Fig. 15 is a cross sectional photograph of a substrate made using this method. The porous layer 60 is 191 ZJB, the interlayer 62 is 720 CWF, and the solid support 64 is code 1737. (Corning Incorporated) The compositions of 191ZJB and 720 CWF are listed below in Table 2 and Table 3. The body was fired at 720C for 2 hours. The 191 ZJB and 720 CWF glasses are expansion matched to 1737 to give minimal bowing of the slide. Chemical durability is also significantly higher than without the bonding layer as determined using a simple tape test after 24 hours in simulated hybridization solution at 70C.

Table #2

Composition of 720 CWF in weight percent:

SiO_2	67.0
Al_2O_3	5.50
Na_2O	2.25
B_2O_3	23.2
CaO	0.40
F	1.00
Li_2O	0.66

Table #3

Composition 191 ZJB in mole percent:

SiO_2	70.32
Al_2O_3	5.12
B_2O_3	17.17
CaO	2.33
BaO	1.79
SrO	0.58
Sb_2O_3	0.20
MgO	0.58
K_2O	1.90

Although the invention has been described in detail for the purpose of illustration, it is understood that such detail is solely for that purpose and variations can be made therein by those of skill in the art without departing from the spirit and scope of the invention which is determined by the following claims:

WE CLAIM:

1. A substrate for the attachment of an array of biomolecules comprising a substantially planar rigid inorganic material having a top surface, said top surface having a plurality of pores disposed therein.

2. The substrate of claim 1 wherein said plurality of pores have a pore radius of between 40Å - 10 μm 40 – 60 angstroms.

3. The substrate of claim 1 wherein said plurality of pores have a pore radius of between 40-60 angstroms.

4. The substrate of claim 1 wherein said top surface is composed of borosilicate glass.

5. The substrate of claim 4 further comprising a layer of cationic polymer electrostatically attached to said top surface.

6. The substrate of claim 4 wherein said cationic polymer is polylysine.

7. The substrate of claim 4 wherein said cationic polymer is gamma-aminopropyltriethoxysilane.

8. The substrate of claim 1 wherein said biomolecules are DNA.

9. A device for performing multiple assays comprising:
a substrate of inorganic material having a substantially planar porous top surface;
A cationic polymer layer bonded to said top surface;
An array of polynucleotides of known predetermined sequence attached to said cationic polymer layer; and

Whereby each said known polynucleotide is attached to a diff rent localized area on said surface.

10. A substrate for the attachment of an array of biomolecules comprising:
a substantially non-porous impermeable bottom layer;
a top layer having a plurality of pores disposed therein;
whereby said top layer is bonded to said bottom layer.

11. The substrate of claim 9 further comprising an index matching liquid infiltrated within said porous surface.

12. The substrate of claim 1 further comprising an index matching liquid infiltrated within said top surface.

13. A method of performing a DNA hybridization assay comprising the step of:
a) providing a substrate having a plurality of pores disposed in a top surface, said surface having a predetermined refractive index;
b) attaching at least one single stranded DNA probe to said top surface;
c) adding a single stranded fluorescently labeled DNA target sequence to said top surface such that said probe sequence and said target sequence are allowed to hybridize;
d) infiltrating into said pores of said top surface, a fluid having a refractive index substantially indentical to said refractive index of said top surface; and
e) measuring said fluorescence emitted from said substrate.

14. The substrate of claim 13 whereby said substrate top surface is porous borosilicate.

15. The method of claim 14 further comprising the step of:
coating said top surface of said substrate with a cationic polymer prior to said attaching step.

16. The substrate of claim 9 further comprising an interlayer disposed between said bottom layer and said top layer.

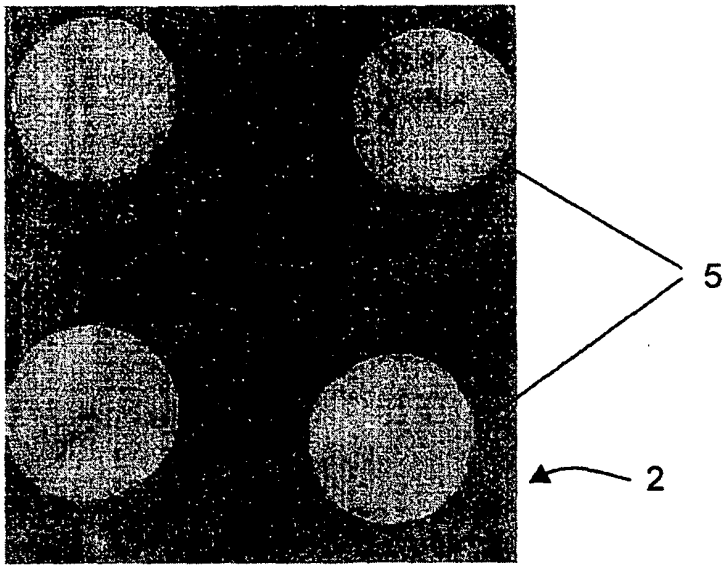


FIG. 1

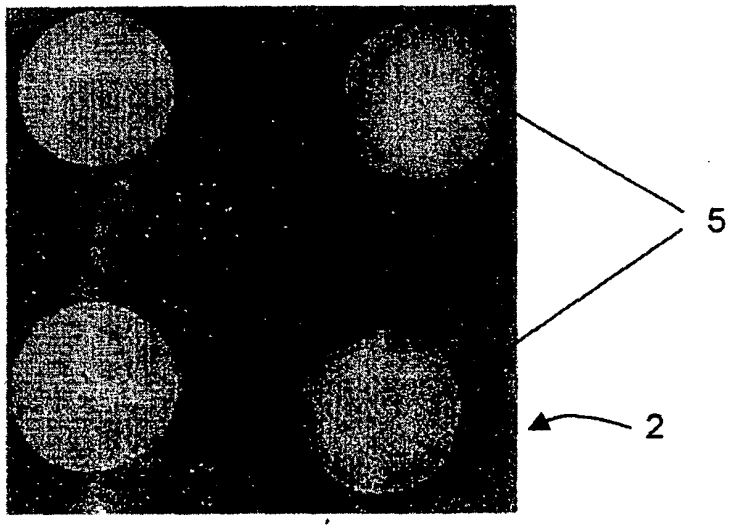
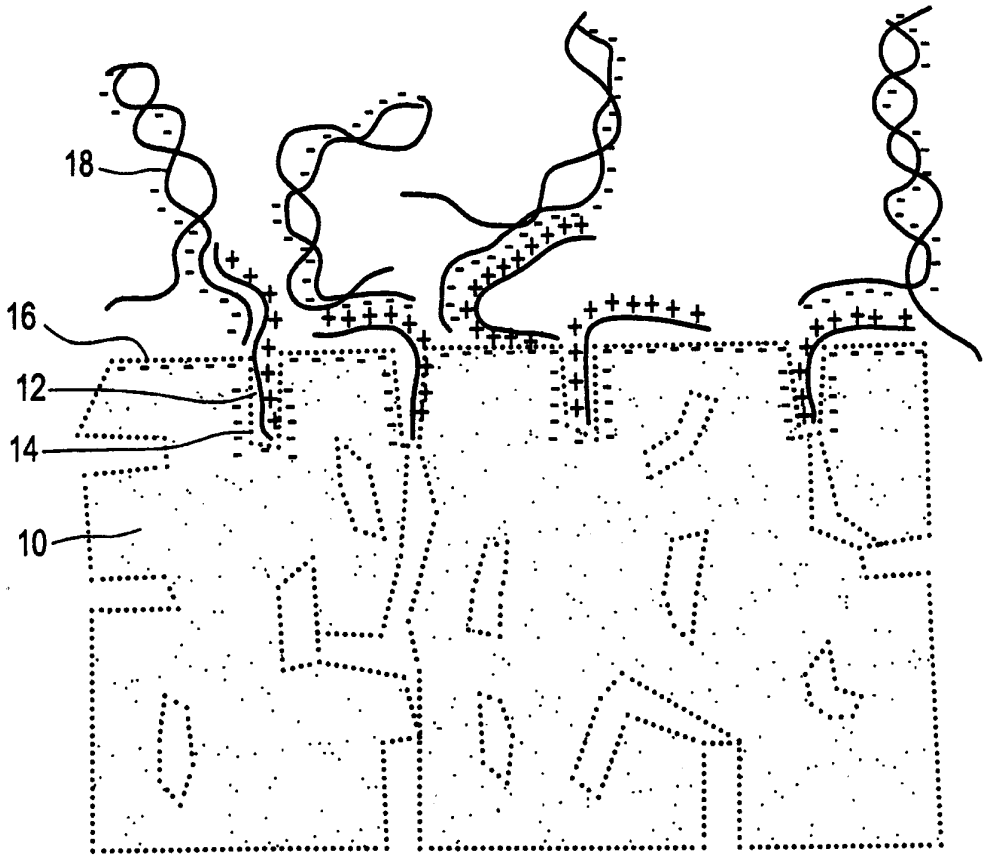


FIG. 2

FIG. 3



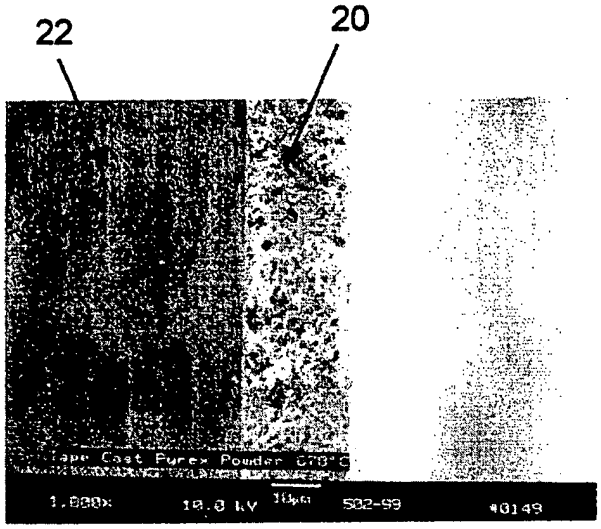


FIG. 4A

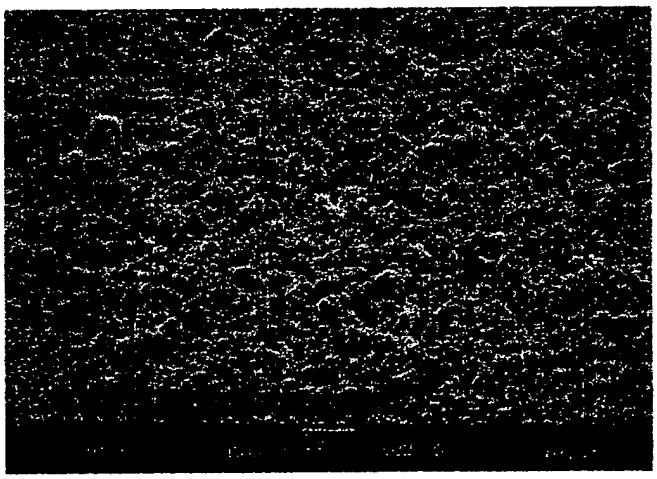


FIG. 4B

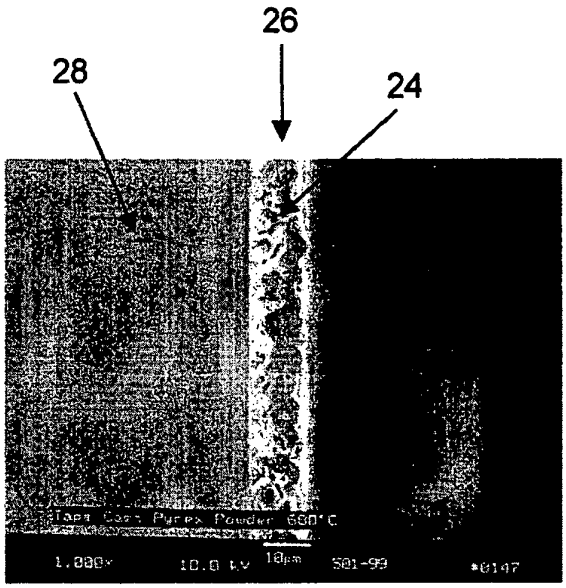


FIG. 5A

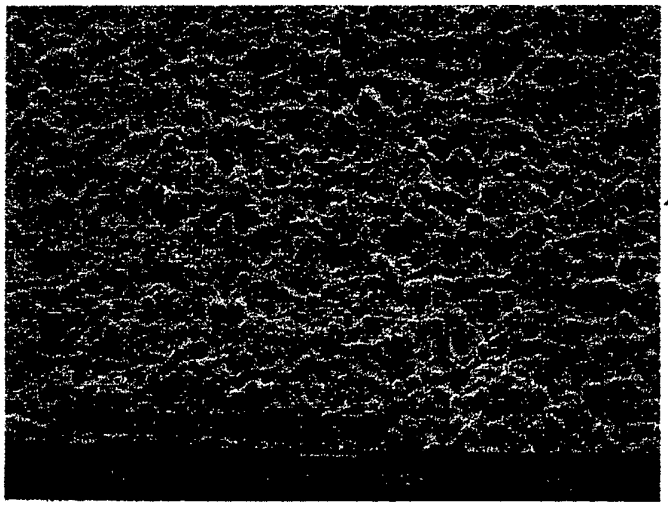


FIG. 5B

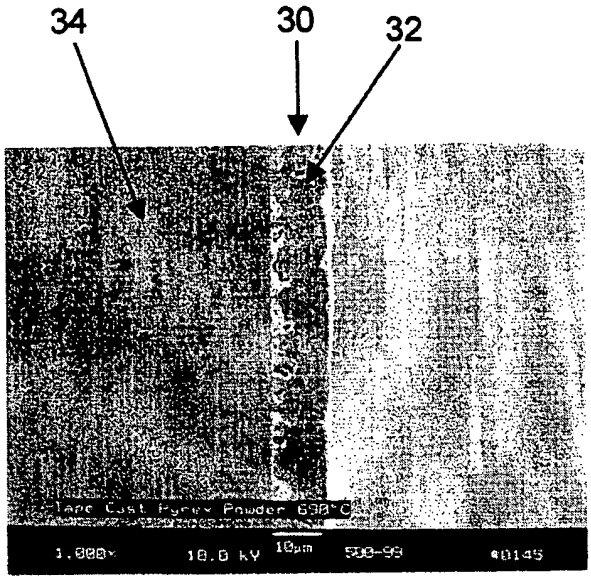

34
30
32
Tape Cast Pyrex Powder 630°C
1.000x 10.0 kV 10µm 500-95 #0145

FIG. 6A

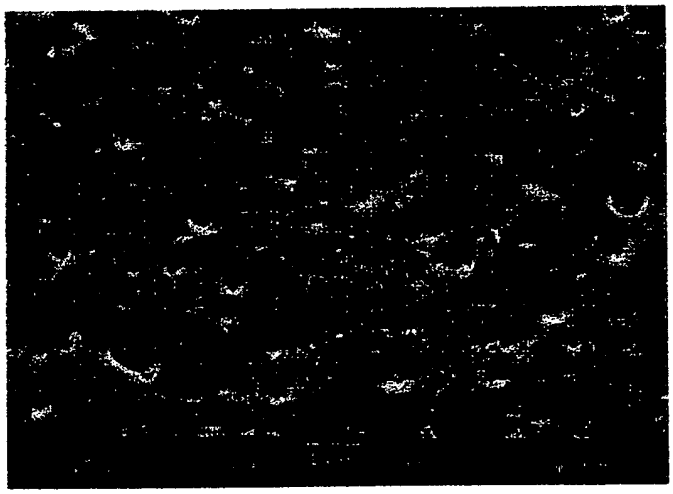

30

FIG. 6B

FIG. 7

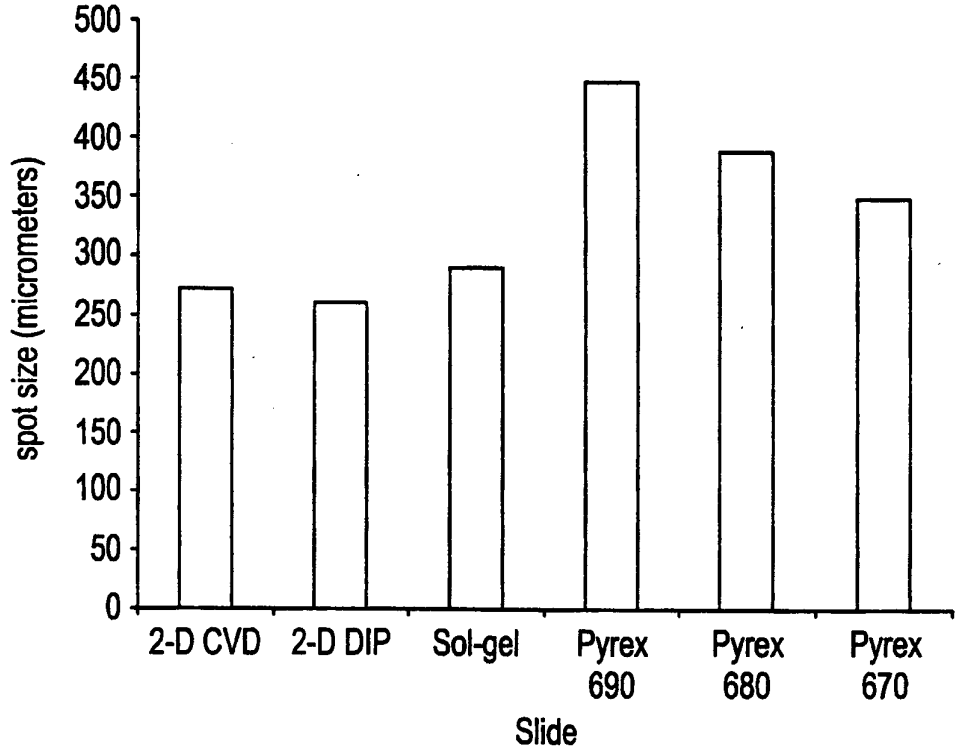

spot size (micrometers)
500
450
400
350
300
250
200
150
100
50
0
2-D CVD
2-D DIP
Sol-gel
Pyrex 690
Pyrex 680
Pyrex 670
Slide

FIG. 8

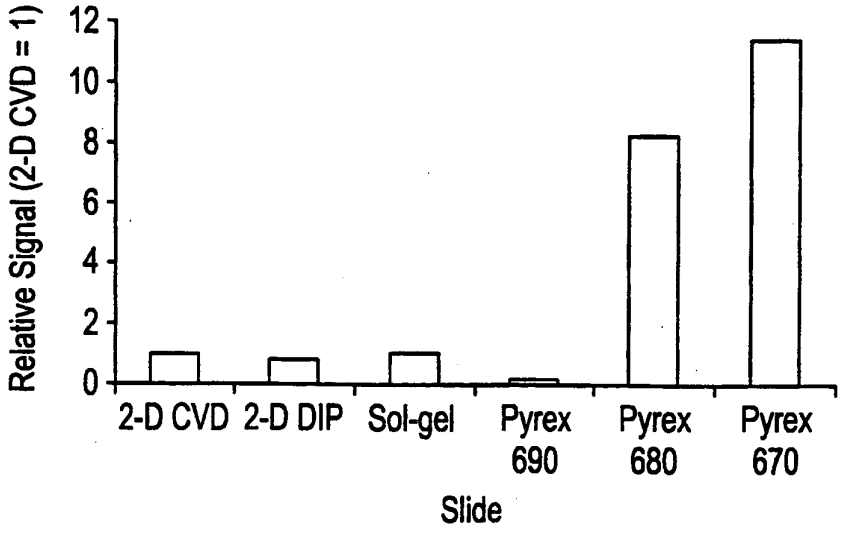

Relative Signal (2-D CVD = 1)
12
10
8
6
4
2
0
2-D CVD
2-D DIP
Sol-gel
Pyrex 690
Pyrex 680
Pyrex 670
Slide

FIG. 9

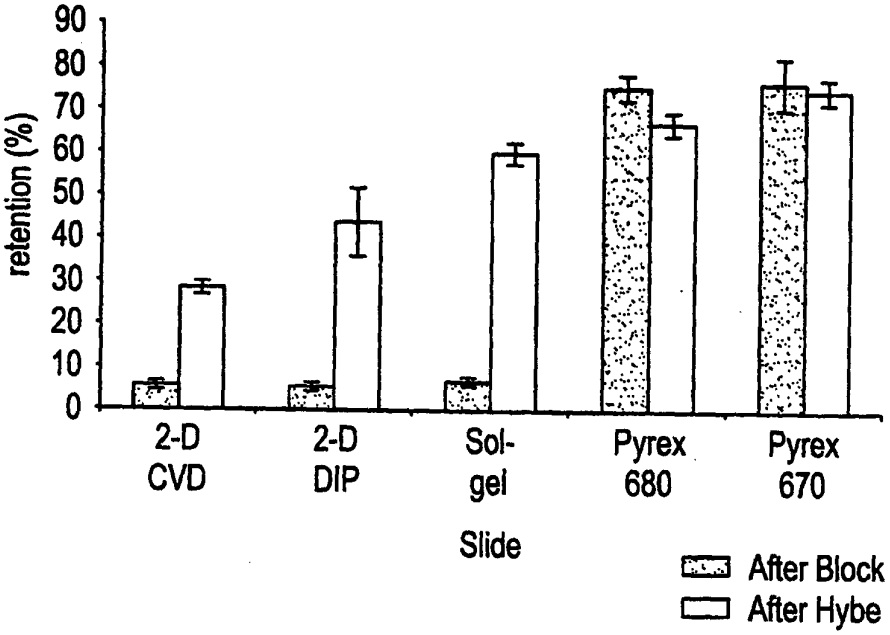

90
80
70
60
50
40
30
20
10
0
retention (%)
2-D CVD
2-D DIP
Sol-gel
Pyrex 680
Pyrex 670
Slide
After Block
After Hybe

FIG. 10

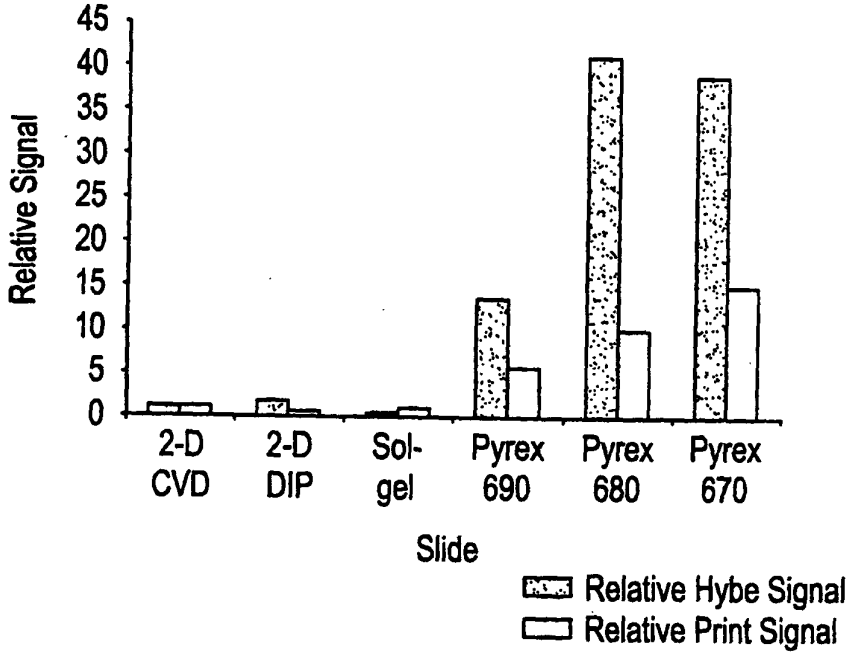

45
40
35
30
25
20
15
10
5
0
Relative Signal
2-D CVD
2-D DIP
Sol-gel
Pyrex 690
Pyrex 680
Pyrex 670
Slide
Relative Hybe Signal
Relative Print Signal

FIG. 11

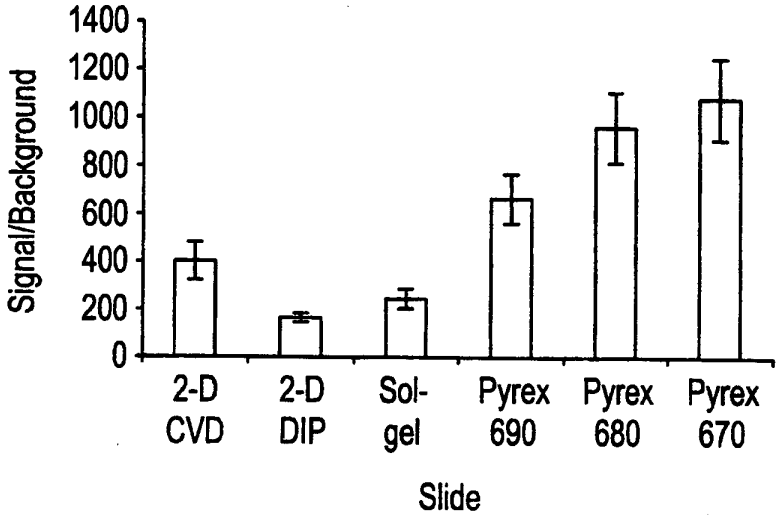
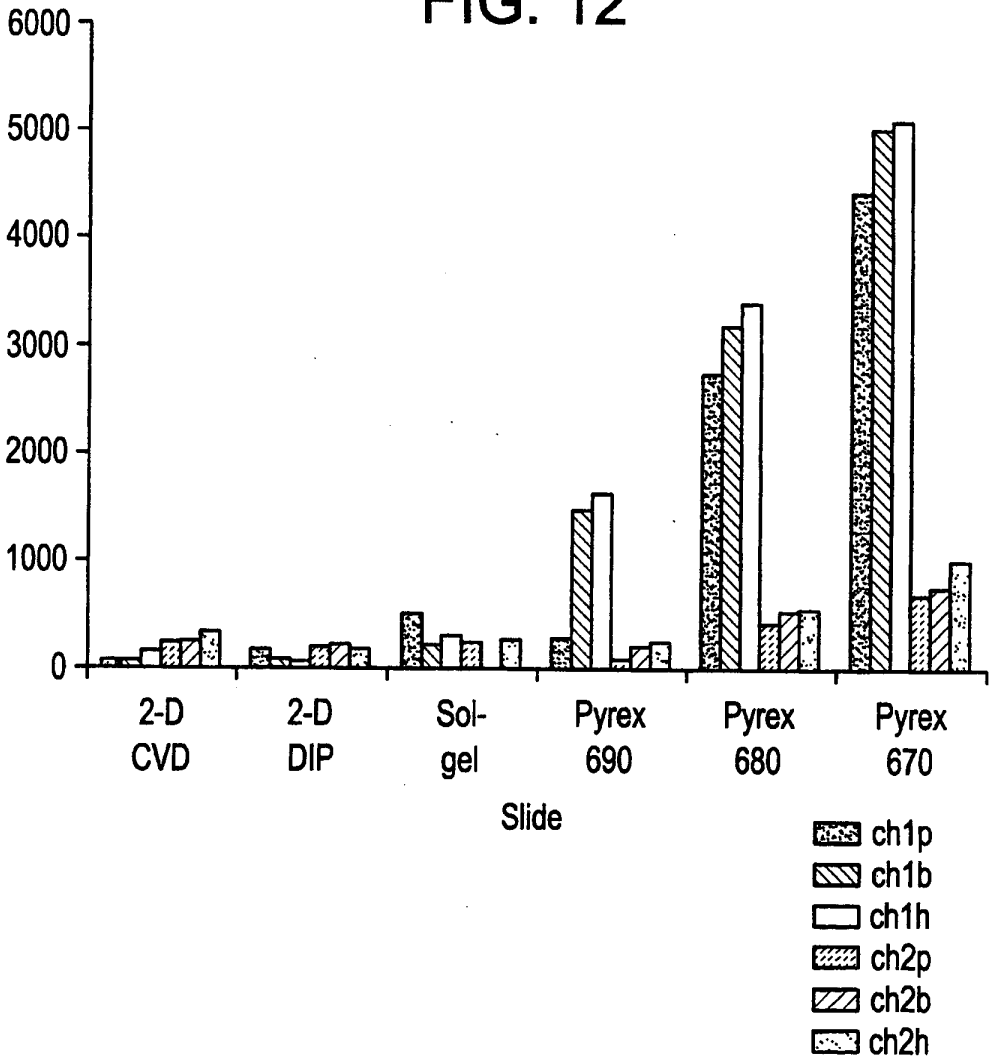

1400
1200
1000
800
600
400
200
0
Signal/Background
2-D CVD
2-D DIP
Sol-gel
Pyrex 690
Pyrex 680
Pyrex 670
Slide

FIG. 12


6000
5000
4000
3000
2000
1000
0
2-D CVD
2-D DIP
Sol-gel
Pyrex 690
Pyrex 680
Pyrex 670
Slide
ch1p
ch1b
ch1h
ch2p
ch2b
ch2h

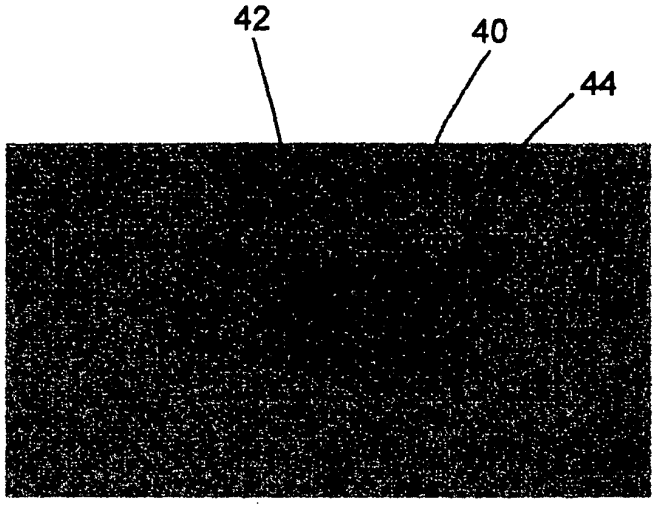


FIG. 13

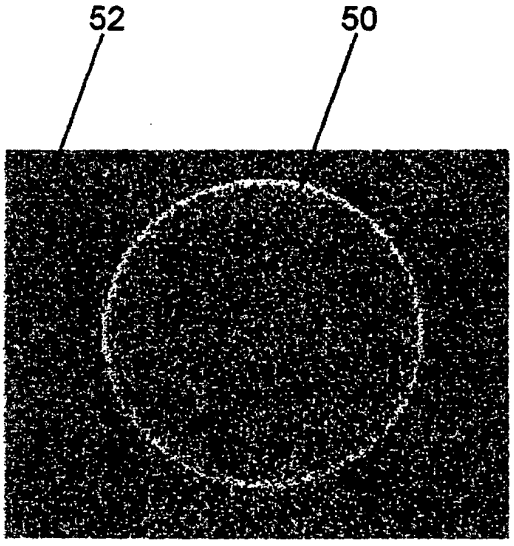


FIG. 14A

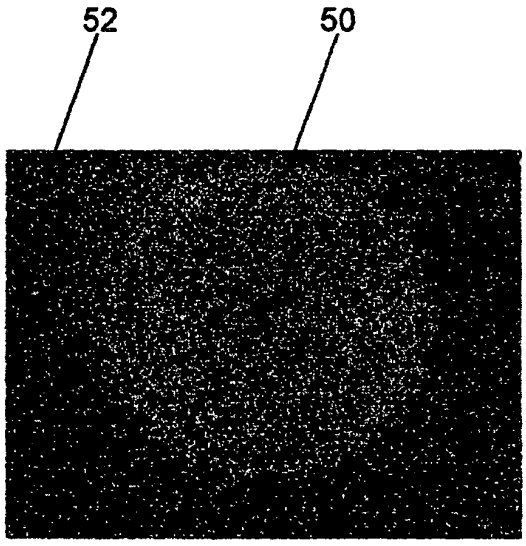
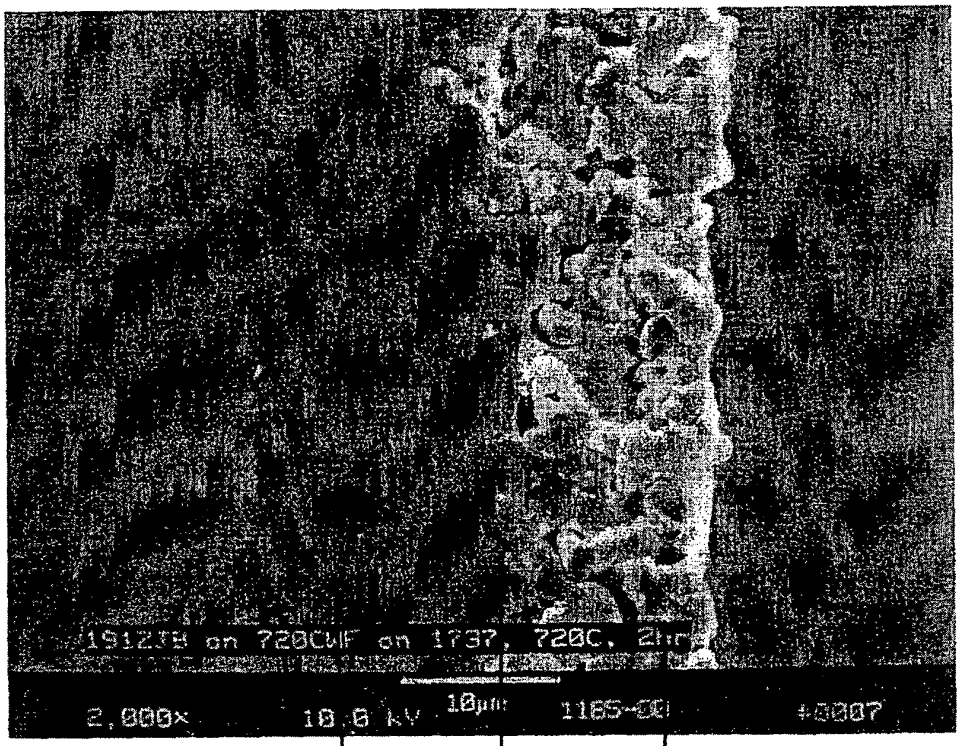


FIG. 14B

FIG. 15



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/23811

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12Q 1/68; G01N 15/06; C12M 1/36;
US CL : 435/6, 287.2, 287.9; 422/68.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 287.2, 287.9; 422/68.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EAST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	US 6,004,752 A (LOEWY et al) 21 December 1999 (21.12.1999) columns 4, 9, and 11.	1-9, 13-16
Y	US 5,843,789 A (NOMURA et al) 01 December 1998 (01.12.1998) see columns 6 and 10.	1-9, 13-16
Y	US 5,770,722 A (LOCKHART et al) 23 June 1998 (23.06.1998) see column 8.	10-12
Y	US 5,807,522 A (BROWN et al) 15 September 1998 (15.09.1998) see column 12.	1-9, 13-16.

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 19 OCTOBER 2000	Date of mailing of the international search report 28 NOV 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer BJ FORMAN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998) ★